THE GENETICS OF ARTEMIA SALINA. III. EFFECTS OF X-IRRADIATION AND OF FREEZING UPON CYSTS[1]

SARANE THOMPSON BOWEN

Department of Biology, San Francisco State College, San Francisco 27, California

One purpose of this study was to find a method of storing brine shrimp cysts without loss of viability. Although Dempster and Hanna (1956) found that they could prevent a gradual decline in hatchability by sealing cysts within a vacuum, their method seemed cumbersome for the small quantities of cysts used in our genetic studies. Another method which appeared promising was that of freezing. Whitaker (1940) reported that cysts could either be maintained in vacuum or subjected to the temperature of liquid air (−190° C.) without affecting the percentage of hatching. However, he did not determine if the nauplii which hatched from frozen cysts could reach maturity. Therefore, experiments were designed in which frozen cysts were tested for both hatchability and ability of the hatched nauplii to reach adulthood.

A second purpose of this study was to find that dose of x-irradiation which would be most efficient for inducing mutations; *i.e.,* the highest dose which would not impair the viability of shrimp emerging from irradiated cysts nor greatly reduce the fertility of inbred stocks derived from them.

The first study of the effects of x-rays on *Artemia* was made by Gajewskaja in 1923 (reviewed by Bonham and Palumbo on pages 155 and 184 of their 1951 paper). Grosch and Sullivan (1955), Grosch and Erdman (1955), and Grosch (1962) showed that when adult shrimp were x-irradiated, 2250 r would sterilize the females but 150,000 r were needed to kill the females. A dose of 200,000 r was required to kill the males. Metalli and Ballardin (1962) also x-irradiated adult shrimps but administered a dose of 1000 r. They found that dominant lethality in the first generation of progeny was much greater for diploid females than for tetraploid females.

Several previous studies have been made of the effects of irradiation upon hatching of *Artemia* cysts: Bonham and Palumbo (1951), Rugh and Clugston (1955), Iwasaki (1958, 1959), Hutchinson and Easter (1960), Easter and Hutchinson (1961), and Engel and Fluke (1962). Only in the study by Bonham and Palumbo was the viability of nauplii observed for a period of more than one

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week after hatching from x-irradiated cysts. These nauplii were not reared to adulthood. Therefore, this paper will report the results of a study of survival of shrimp hatched from x-irradiated cysts.

Materials and Methods

Source of the cysts

Three collections of dry cysts from bisexual races of *Artemia* were studied. *Sample A* was collected from solar evaporating ponds in San Francisco Bay in the summer of 1960. In the summer of 1961, this sample was divided into ten lots, five of which were frozen. It was obtained through the courtesy of Mr. Maurice Rakowicz, Brine Shrimp Sales Co., Inc., in Hayward, California. *Sample R* was collected from ponds in San Francisco Bay in the summer of 1957. In the spring of 1959 it was divided into two lots, one of which was frozen. It was obtained from the San Francisco Aquarium Society. *Sample U* was collected from Great Salt Lake, Utah, in the summer of 1957. In the fall of 1960 it was divided into two lots, one of which was frozen. It was obtained through the courtesy of Mr. C. C. Sanders, Sanders Brine Shrimp Co., Ogden, Utah.

Irradiation

Cysts from sample A were irradiated in June, 1961. The x-ray source was a General Electric Maxitron 250 operated with a 0.0761 mm. Cu filter (HVL 0.58 mm. Cu) at 250 KeV, 30 ma, at a target distance of 16.5 cm. The dose rate was 3024 r/min. in air. The HVL (half-value layer) was measured with a Victoreen Count Rate Meter and a Victoreen #601 Probe. The output was measured with a Hold #5 chamber and Victoreen R-Meter. The dry cysts were in size 00 gelatin capsules ($\frac{1}{4}''$ outer diameter and $\frac{3}{4}''$ long) at the time of irradiation. Immediately thereafter, the cysts were transferred to dry glass bottles (2.5 ml. capacity).

Storage of cysts

The cyst samples were stored in glass bottles, either in a freezer ($-19°$ to $-24°$ C.) or in darkness at room temperature (20° to 28° C.). The room temperature in our laboratory fluctuates within the narrow range of 21° to 23° C. for about 340 days of each year.

Cyst viability tests

The *Artemia* cyst is actually an encysted blastula, about 200 μ in diameter. When a viable dry cyst is immersed in water, it completes gastrulation. Excystment takes place in two stages. First, the embryo, enclosed within a transparent membrane, emerges from the ruptured shell. A few hours later, it hatches out of the membrane as a free-swimming phototropic nauplius. This process has been described in detail by Whitaker (1940) and by Myint (1956).

The criteria of cyst viability were: (1) *emergence,* (2) *hatching,* and (3) *motility.* Tests were made by counting the number of cysts yielding (1) partially

emerged embryos, (2) nauplii free of their enclosing membranes, and (3) nauplii with motile second antennae.

In the earlier methods, *Method A* was used: five cysts were placed in each of 20 shell vials, one-half inch of sea water was added, and the numbers emerged and hatched were recorded. In 1963, *Method B* was adopted. Cysts were scattered on the surface of sea water agar in petri dishes. (This medium was made by adding eight grams of agar to a liter of sea water.) Sterile techniques were not used. The value for each cyst sample was based on data from at least two agar plates. Records were kept on emergence, hatching, and motility. In both methods, counts were made on the second and third days after hydration of the cysts. In method B, the counts could be made more rapidly but hatching and motility values were lower and more variable than in method A. In all experiments reported in this paper, the criterion of cyst viability was hatching when method A was used and emergence when method B was used.

Survival tests

A single layer of cysts was sprinkled onto the bottom of a 250-ml. beaker and 50 ml. of filtered sea water were added. Forty-eight hours later, the nauplii were transferred into the culture medium (50 grams of NaCl per liter of filtered sea water). Two nauplii were placed in each shell vial (21 mm. diameter and 70 mm. high) containing about five ml. of the culture medium. Brewer's yeast was added according to the standard feeding schedule described previously (Bowen, 1962). All survival values were arbitrarily taken to be the number of shrimp alive three weeks after hatching. At this time, all controls had reached at least the twelfth instar of Heath (1924); that is, they could be classified according to sex.

Search for mutations

The shrimp which hatched from the irradiated cysts and four generations of their progeny were examined for mutations throughout the summer and fall of 1961. They were reared in shell vials, fed according to the standard feeding schedule described previously (Bowen, 1962), and were examined without anesthetization under a dissecting microscope (7 ×). All matings consisted of a single pair of shrimp. Pedigree records were maintained on the progeny.

Results and Discussion

1. *Effect of freezing upon viability*

The three samples, A, R and U, were each divided into two lots. One lot was placed in the freezer while the other was stored in the dark at room temperature. The viability of both lots was tested at intervals. Whenever a sealed bottle was removed from the freezer, it was allowed to reach room temperature before it was opened. This precaution was taken to prevent condensation of moisture upon the cold cysts which might deteriorate with repeated hydration and freezing.

The data in Table I indicate that frozen storage does not impair the hatching percentage of cysts. On the contrary, it prevents the decline in viability associ-

TABLE I

Viability of cysts stored at two different temperatures

Cyst sample	Source of cysts	Length of storage	Viability test method and criterion of viability	Non-frozen storage		Frozen storage	
				Viable/total	%	Viable/total	%
A	San Francisco Bay, California	1 day	A. hatching	332/400	83	304/400	76
		12 days	A. hatching	156/200	78	140/200	70
		22 months	B. emergence	1626/2400	68	1778/2400	74
		25 months	B. emergence	965/1600	60	1223/1600	76
R	San Francisco Bay, California	6 months	A. hatching	887/1790	50		
		13 months	A. hatching	31/60	51	39/60	65
		23 months	A. hatching	19/100	19	63/100	63
		25 months	A. hatching	25/200	12	146/200	73
		4 years	B. emergence	2/1000	0.2	662/1000	66
U	Great Salt Lake, Utah	9 months	A. hatching	32/100	32	63/100	52
		3 years	B. emergence	1/700	0.1	382/700	55

ated with aging at room temperature. In the case of samples R and U, the viability of the unfrozen cysts deteriorated markedly until the percentage of viable cysts was less than 1%. However, in the case of sample A, the viability of the unfrozen cysts did not show a significant decline. This disparity probably can be accounted for by the fact that at the time the three samples were each divided into two lots to be stored at different temperatures, samples A, R and U had aged at room temperature for one, two and three years, respectively. It is possible that decline in viability occurs only after cysts have aged for two years at room temperature. The yearly tests of the viability of these cyst samples will be continued in order to determine if this is the case. The decline in viability of samples R and U seems more extreme than that reported by other authors. For example, Dempster and Hanna (1956) reported that after three years' storage, their control cysts had hatchabilities of about one-fourth to one-sixth of the values obtained

TABLE II

Survival of nauplii which have hatched from cysts stored at two different temperatures. (Survival scored as number of shrimp alive three weeks after hatching. The asterisk indicates that hatchability had fallen so low that adequate numbers of nauplii could not be obtained for a survival test.)

Cyst sample	Source of cysts	Length of storage	Non-frozen storage		Frozen storage	
			Survivors/total	%	Survivors total	%
R	San Francisco Bay, California	2 years	37/100	37	29/100	29
		4 years	*		70/200	35
A	San Francisco Bay, California	12 days	37/100	37	43/100	43
		22 months	114/200	57	141/200	70

for the initial control sample or of samples stored *in vacuo*. Clegg (1962) obtained 4% hatching from a sample of cysts stored for ten years.

In Table II, it can be seen that freezing of cysts did not impair the viability of the nauplii hatching from these cysts. The only valid comparisons in Table II are comparisons of values in the same horizontal line. This is due to the fact that whenever one cyst sample is tested for nauplius survival, the tests set up at different times yield markedly different results. Additional evidence for this will be seen in Table IV.

In the last experiments on sample R (Tables I and II), the frozen dry cysts had been through four cycles of freezing and thawing. Evidently, this did not damage either the cysts or the shrimp hatching from them. This hardiness may be attributed to the low water content of the cysts (which contain about 8% water removable by vacuum treatment, according to Engel and Fluke, 1962) and their relatively high content of glycerol (about 5%, according to Clegg, 1962).

2. *Effect of x-irradiation upon viability*

Cysts from sample A were divided into ten lots. Two lots were exposed to each of the five levels of irradiation: 0, 400, 2000, 10,000, and 50,000 r. After irradiation, they were transferred into glass bottles and one lot (from each dose) was stored in the dark at room temperature and the other was frozen. Preliminary tests indicated that the 400 r and 2000 r doses did not significantly affect either hatchability of cysts or survival of nauplii. Therefore, subsequent tests were made only of cysts receiving 10 and 50 kr.

In Table III, each value from Experiments 4, 5 and 6 is based on data from two to four agar plates. The difference between control and irradiated values can be assigned to experimental error (discrepancies between plates testing the same lot of cysts). Thus, the 50-kr dose had no significant effect upon cyst viability. This finding is in agreement with the report of Rugh and Clugston (1955) that a 100-kr dose of x-irradiation delivered to dry *Artemia* cysts did not decrease the percentage hatching.

TABLE III

Viability of x-irradiated cysts. (All cysts from sample A from San Francisco Bay.)

Expt.	Time between irradiation and test for viability	Storage of cysts after irradiation	Viability test method	0 Roentgens		10,000 Roentgens		50,000 Roentgens	
				Viable/total	%	Viable/total	%	Viable/total	%
1	1 day	non-frozen	A	169/200	84	175/200	88	88/100	88
2	13 days	non-frozen	A	217/300	72	215/300	72		
3	52 days	non-frozen	A	164/200	82			127/200	64
4	21 months	non-frozen	B	404/600	67			397/600	66
5	22 months	non-frozen	B	565/800	71	533/800	67	528/800	66
	22 months	frozen	B	618/800	77	621/800	78	539/800	67
6	25 months	non-frozen	B	488/800	61			477/800	60
	25 months	frozen	B	592/800	74			631/800	79

TABLE IV

Survival of nauplii which have hatched from x-irradiated cysts. (All cysts from sample A from San Francisco Bay. Survival scored as number of shrimp alive three weeks after hatching.)

Experiment	Time between irradiation and test for survival	Storage of cysts after irradiation	0 Roentgens survivors/total	10,000 Roentgens survivors/total	50,000 Roentgens survivors/total
1	1 day	non-frozen	5/50	11/50	0/50
2	13 days	non-frozen	32/100	29/100	2/100
3	52 days	non-frozen	63/100	73/100	0/100
4	22 months	non-frozen	56/100	58/100	0/100
	22 months	frozen	64/100	77/100	0/100
	Total		220/450	248/450	2/450
	% Survival		49%	55%	0.4%

Inspection of the data in Table IV reveals the great fluctuation in survival values for nauplii from a single cyst sample when the survival tests are set up at different times. For example, the survival values for non-irradiated nauplii ranged from 10% to 64%. Despite the great fluctuation from one experiment to another, it is clear that, in each of the four experiments, survival of the nauplii from the 50-kr dose was markedly reduced. When the results were totaled, it was found that of the 450 nauplii which hatched from control cysts, 220 reached adulthood and survived to the end of the third week. Of these, 107 were males and 113 were females. Of the 450 nauplii hatching from cysts receiving 10 kr, 248 survived: 128 males and 120 females. In both control and 10-kr groups, the shrimp were 6 to 10 mm. long and all had reached the twelfth instar of Heath (1924). Of the 450 nauplii from the 50-kr group, only two survived: a male and a female. They were the most mature of all the metanauplii in the 50-kr group, yet the male was only 5 mm. and the female only 3.5 mm. in length. Their second antennae were similar to those shown by Heath (1924) to be typical of the eighth and ninth instars for the female and male, respectively. In both shrimp, one eye was larger than the other. The abdomen of the female was constricted and poorly segmented. Both animals failed to mature further and died a few days after the termination of the experiment.

Throughout the survival tests, the shrimp in the 50-kr dose group were smaller and had lower viability than those in both the control and the 10-kr groups. The shrimp in the latter two groups were not significantly different in maturity or viability. The numbers of surviving shrimp are plotted in Figure 1.

It is difficult to compare these survival tests with those of Bonham and Palumbo (1951) because the shrimp in their experiments were maturing at a much slower rate. For example, Table 9 in their paper shows that at the end of eleven days, none of their metanauplii had attained a length of 1 mm. (even in the control group). At this age, all of our controls exceeded that length. Bonham and Palumbo estimated that the LD_{50} for dry *Artemia* cysts was about 50 kr when the experiment was terminated at the end of two weeks (page 185).

If one considers the entire life span of *Artemia*, the data in the present investigation show that the LD_{50} must lie between 10 and 50 kr.

The data in Tables III and IV and in Figure 1 indicate that although the 50-kr dose does not significantly affect the development of the shrimp through the stages of gastrulation and hatching, its effect upon development through the

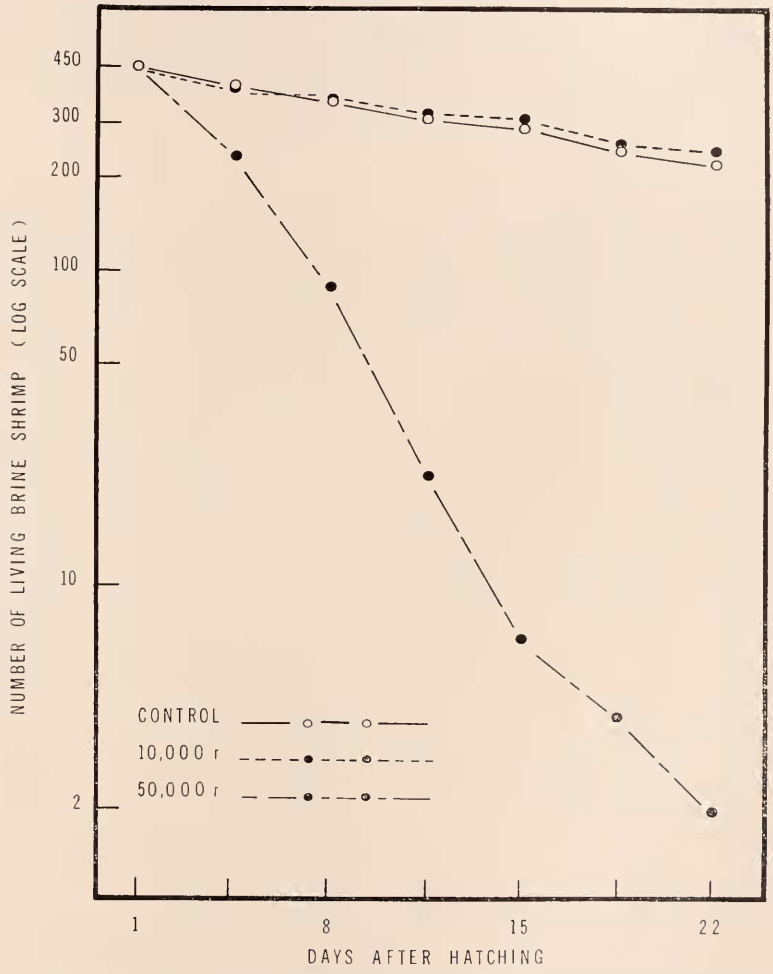

FIGURE 1. Survival of *Artemia* nauplii which have hatched from cysts receiving 0, 10, or 50 kr of x-ray irradiation.

first few instars is extreme. It is surprising to find that a lethal 50-kr dose administered to the encysted blastula does not kill the animal during the earlier stages of gastrulation and embryonic differentiation. This remarkable phenomenon may be explained by the report of Nakanishi *et al.* (1962) that *Artemia* blastulae differentiate without cleavage. These authors found no mitotic figures

during gastrulation; only a few figures were seen at the time the embryo emerged from the cyst. Post-irradiation cell death rarely occurs until the time that one or several successive mitotic divisions have occurred (reviewed by Lea, 1946; by Puck *et al.*, 1957; by Puck, 1960; and by Jacobson, 1962). For this reason, radiation damage has little chance to express itself in *Artemia* during embryonic development.

The brine shrimp cyst has often been cited as an outstanding example of radioresistance. A small portion of this resistance may be attributed to the fact that tests of emergence or hatching have been used as the indices of radiation damage. However, these tests are made at a time when only a few successive cell divisions have occurred following irradiation.

3. *Search for mutations*

The irradiated shrimp and their progeny were examined in the hope of finding visible (that is, non-lethal) mutations. The abnormal appearance of the two survivors of the 50-kr dose has been described in Part 2. No abnormalities were seen in the shrimp which hatched from cysts given 400, 2000, or 10,000 r doses. Single-pair matings were made up among the survivors of the 2000-r dose and also among the survivors of the 10,000-r dose. These pairs constituted the X-0 generation and their progeny were the X-1 generation. Siblings from the X-1 were mated to produce an X-2 generation. In a similar fashion, sibling matings were continued to produce X-3 and X-4 generations.

Because the dry cysts contain blastulae and because these blastulae differentiate without cleavage (Nakanishi *et al.*, 1962), the nauplii which emerge from irradiated cysts are somatic mosaics. Theoretically, every cell in an emerging nauplius might carry a mutation at a different locus. In adult shrimp of the X-0 generation, dominant mutations would be expressed as patches of abnormal tissue. In the following generations, the shrimp would not be expected to be mosaics. The X-1 generation would show the effects of irradiation-induced dominant mutations. Because females are heterogametic (Bowen, 1963), the X-1 females would show effects of new mutations located on the differential segments of the X and Y chromosomes. It would not be possible to discover mutant traits governed by the irradiation-induced autosomal recessive genes until the X-2 or later generations. Similarly, traits such as the white eye of *Artemia* (Bowen, 1963), which are partially sex-linked, would not be seen until the X-2 or later generations.

The inbred stocks derived from survivors of the 10-kr dose showed considerable loss of fertility which was probably due to x-ray-induced lethals and semilethals. Whenever a deviant shrimp was found in 10 kr progeny, it was outcrossed to the control stock; its siblings and parents were inbred in the hope of producing more progeny with the mutant phenotype. Several aberrant traits were observed in the progeny of shrimp from the 10 kr and 2 kr groups. There were five independent occurrences of absence of setae on the distal lobe of the legs. There were three independent occurrences of swollen abdomen. Other traits were bent abdomen, kidney-shaped eyes, and a bump on the seventh segment of the abdomen. None of these was sufficiently viable for genetic experiments. It is probable that these deformities were induced by irradiation because these departures from the wild-type have not been found in non-irradiated *Artemia*.

A shrimp with garnet eye color appeared in the X-2 generation of the progeny of two shrimp from the 10-kr dose group. It was outcrossed to the control stock and more garnet-eyed offspring were recovered in the F_2. The genetic segregation data have appeared in an unpublished thesis (Hanson, 1963) and will be published in a later paper in this series. Garnet eye color is determined by a recessive autosomal gene. The eye color of homozygous shrimp becomes progressively lighter with age. At three weeks of age, the eyes are garnet (reddish-brown). At six to eight weeks, the eyes are transparent except for a few scattered round cells filled with garnet pigment.

Summary

1. Samples of dry brine shrimp cysts were each divided into two lots: one stored at room temperature and the other stored in a freezer (−19° to −24° C.). Viability of the frozen cysts remained unchanged whereas that of the unfrozen cysts declined. Nauplii hatching from frozen cysts had normal viability. It is evident that frozen storage is superior to unfrozen storage if *Artemia* cysts are to be kept for a period of several years.

2. Dry cysts from a California race were exposed to x-irradiation. Although the 50-kr dose had no significant effect upon the viability of the cysts, it greatly reduced the viability of the nauplii which hatched from these cysts; none of these nauplii were able to live to maturity. It was surprising to find that a 50-kr dose administered to the encysted blastula did not kill during the stages of gastrulation or embryonic differentiation but instead killed during the later developmental stages (the second through the eighth instars). This can probably be explained by the finding of Nakanishi *et al.* that *Artemia* blastulae differentiate without cleavage.

3. The effect of x-rays upon viability was the same when cysts were hydrated and tested immediately after irradiation as when they were tested after two years' storage at room temperature or in a freezer.

4. Irradiated shrimp from the 2-kr and 10-kr doses and four generations of their inbred progeny were examined in hope of finding visible mutant traits. Although many aberrant characteristics were observed, only one viable pure-breeding mutant stock was developed. It is characterized by garnet eye color which is determined by a recessive autosomal gene. The first garnet-eyed shrimp was found in the second generation of the progeny of two shrimp which hatched from cysts given 10 kr of x-irradiation.

5. The 10-kr dose did not impair the viability of cysts or the survival of hatched nauplii to adulthood. It did not completely depress the fertility of inbred progeny derived from the irradiated generation. Therefore, it appears to be an efficient dose for inducing visible mutations for use in genetic studies.

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